Mechanisms of Signal Transduction:

Pretreatment of Acetylsalicylic Acid Promotes Tumor Necrosis Factor-related Apoptosis-inducing Ligand-induced Apoptosis by Down-regulating *BCL-2* Gene Expression

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Pretreatment of Acetylsalicylic Acid Promotes Tumor Necrosis Factor-related Apoptosis-inducing Ligand-induced Apoptosis by Down-regulating *BCL-2* Gene Expression*

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Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) has been shown to be selective in the induction of apoptosis in cancer cells with minimal toxicity to normal tissues. However, not all cancers are sensitive to TRAIL-mediated apoptosis. Thus, TRAIL-resistant cancer cells must be sensitized first to become responsive to TRAIL. In this study, we observed that pretreatment by acetylsalicylic acid (ASA) augmented TRAIL-induced apoptotic death in human prostate adenocarcinoma LNCaP and human colorectal carcinoma CX-1 cells. Western blot analysis showed that pretreatment of ASA followed by TRAIL treatment activated caspases (8, 9, and 3) and cleaved poly(ADP-ribose) polymerase, the hallmark feature of apoptosis. Most interestingly, at least 12 h of pretreatment with ASA was prerequisite for promoting TRAIL-induced apoptosis and was related to down-regulation of *BCL-2*. Biochemical analysis revealed that ASA inhibited NF-κB activity, which is known to regulate *BCL-2* gene expression, by dephosphorylating IκB-α and inhibiting IKKβ activity but not by affecting the HER-2/neu phosphatidylinositol 3-kinase-Akt signal pathway. Overexpression of *BCL-2* suppressed the promotive effect of ASA on TRAIL-induced apoptosis and changes in mitochondrial membrane potential. Taken together, our studies suggested that ASA-promoted TRAIL cytotoxicity is mediated through down-regulating *BCL-2* and by decreasing mitochondrial membrane potential.

Tumor cells develop resistance to apoptotic stimuli induced by various therapeutic agents, such as drugs, irradiation, and immunotherapy, because most of their primary cytotoxic effects are through apoptosis (1, 2). After the initial response to these therapies, tumor cells develop resistance and/or are selected for resistance to apoptosis. Therefore, new therapeutic strategies are needed to reverse resistance to apoptosis.

Recent studies have also revealed that TRAIL,[2] which is constitutively expressed on murine natural killer cells in the liver, plays an important role in surveillance of tumor metastasis (3). The apoptotic signal of TRAIL is transduced by binding to the death receptors TRAIL-R1 (DR4) and TRAIL-R2 (DR5), which are members of the tumor necrosis factor-α receptor superfamily. Both DR4 and DR5 contain a cytoplasmic death domain that is required for TRAIL receptor-induced apoptosis. TRAIL also binds to TRAIL-R3 (DcR1) and TRAIL-R4 (DcR2), which act as decoy receptors by inhibiting TRAIL signaling (4–7). Unlike DR4 and DR5, DcR1 does not have a cytoplasmic domain, and DcR2 retains a cytoplasmic fragment containing a truncated form of the consensus death domain motif (8). Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a cytotoxic molecule that has been shown to exert, selectively, anti-tumor cytotoxic effects both *in vitro* and *in vivo* with minimal toxicity to normal tissues (9, 10).

TRAIL has been considered a new therapeutic agent, and preclinical studies demonstrate its antitumor activity alone or in combination with drugs (10–13). However, many tumor cells have been shown to be resistant to TRAIL (14, 15). Several researchers have reported that TRAIL resistance can be overcome by various sensitizing agents like chemotherapeutic drugs (16, 17), cytokines (18), and matrix metalloprotease inhibitors (19) that are able to render TRAIL-resistant tumor cells sensitive to TRAIL apoptosis.

In recent studies, nonsteroidal anti-inflammatory drugs (NSAIDs), such as acetylsalicylic acid (aspirin; ASA), have been used as chemopreventive agents of cancers to induce apoptosis or to reduce the incidence of tumor formations in a variety of organs, *i.e.* colon (20), lung (21), stomach (22), and colorectum (23). ASA is known to act by directly suppressing the cyclooxygenase enzyme (COX-1 and COX-2), the rate-limiting enzyme catalyzing the biosynthesis of prostaglandins, thereby blocking the production of proinflammatory prostaglandins. ASA was also shown to be effective in the inhibition of ultraviolet radiation and carcinogen-induced tumor formations in animal models (24, 25).

In this study, we examined whether ASA in combination with TRAIL increases TRAIL-induced apoptotic death in TRAIL-resistant human cancer cells. We hypothesized that pretreatment with ASA enhances TRAIL-induced apoptosis by promoting the mitochondria-dependent apoptotic pathway. Our studies demonstrate that ASA augments TRAIL-induced apoptosis by down-regulating *BCL-2* gene expression and by decreasing mitochondrial membrane potential, which subsequently leads to an increase in caspase activation.

EXPERIMENTAL PROCEDURES

Reagents—ASA, NS-398, indomethacin, sulindac sulfide, and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone were obtained from Sigma. Tetramethylrhodamine methyl ester (TMRM) was purchased from Molecular Probes (Eugene, OR). Anti-Bcl-X_L, anti-phospho(Tyr-

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[2] The abbreviations used are: TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; DcR1, decoy receptor 1; DcR2, decoy receptor 2; DR4, death receptor 4; DR5, death receptor 5; ASA, acetylsalicylic acid; DTT, dithiothreitol; TMRM, tetramethylrhodamine methyl ester; PARP, poly(ADP-ribose) polymerase; PBS, phosphate-buffered saline; NSAID, nonsteroidal anti-inflammatory drugs; TUNEL, terminal deoxynucleotidyltransferase-mediated nick end-labeling; PI3K, phosphatidylinositol 3-kinase; siRNA, small interfering RNA; PDK1, phosphoinositide-dependent kinase-1; RT, reverse transcription; IKK, IκB kinase.

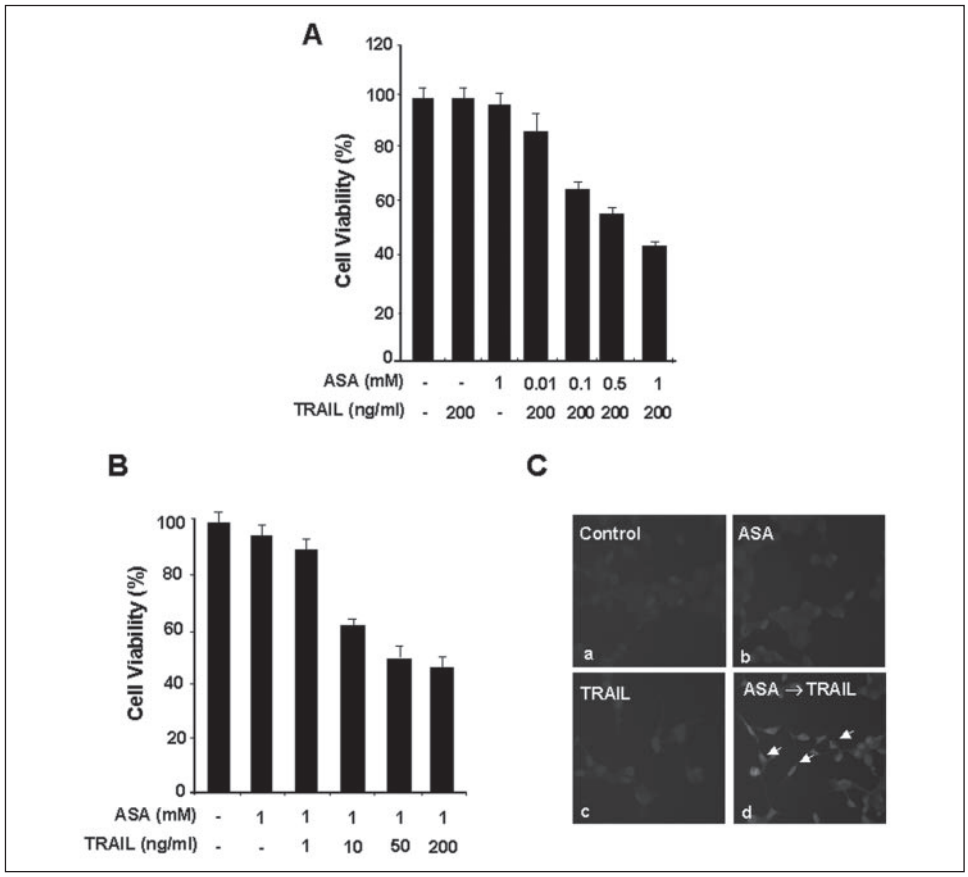

FIGURE 1. **Effect of pretreatment of acetylsalicylic acid on TRAIL-induced cytotoxicity in human prostate adenocarcinoma LNCaP cells.** *A,* cells were pretreated with various concentrations of ASA (0.01–1 mM) for 20 h and treated with/without 200 ng/ml TRAIL for 4 h. Cell survival was determined by the trypan blue exclusion assay. *Error bars* represent the means ± S.E. from three separate experiments. *B,* cells were pretreated with 1 mM ASA for 20 h and treated with/without various concentrations of TRAIL (1–200 ng/ml) for 4 h. Cell survival was determined by the trypan blue exclusion assay. *Error bars* represent the means ± S.E. from three separate experiments. *C,* cells were pretreated with 1 mM ASA for 20 h and treated with/without 200 ng/ml TRAIL for 4 h. After treatment, apoptosis was detected by the TUNEL assay. Apoptotic cells are indicated by *arrows*. *a,* untreated control; *b,* ASA only; *c,* TRAIL only; *d,* ASA → TRAIL.

508)-PI3K, anti-caspase-3, anti-caspase-9, anti-histone H1, anti-IKKα, and anti-IKKβ antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-HER-2/neu antibody was purchased from DakoCytomation (Carpinteria, CA). Anti-DR5 and anti-DcR2 bodies were purchased from StressGen (Victoria, British Columbia, Canada). Anti-DR4 antibody, anti-caspase-8 antibody, and GST-IκBα protein were purchased from Upstate Biotechnology (Lake Placid, NY). Anti-COX-2 antibody was purchased from Cayman Chemical (Ann Arbor, MI). Anti-phospho(Tyr-1248)-HER-2/neu, anti-phospho(Ser-473)-Akt, anti-Akt, anti-phospho(Ser-32)-IκB-α, anti-IκB-α, anti-phospho(Ser-241)-PDK-1, and anti-PDK-1 antibodies were purchased from Cell Signaling (Beverly, MA). Anti-cIAP-1 and anti-cIAP-2 antibodies were purchased from R&D Systems (Minneapolis, MN). Anti-FLIP antibody was purchased from Calbiochem. Anti-PARP was purchased from Biomol. Anti-Bcl-2 and anti-actin antibodies were purchased from ICN (Costa Mesa, CA).

Cell Culture and Survival Assay—Human prostate adenocarcinoma LNCaP and DU-145 cell lines, human colorectal carcinoma CX-1 cell line, and normal prostate YPEN cell line were obtained from the American Tissue Type Culture Collection (Manassas, VA). LNCaP, CX-1, and YPEN cells or DU-145 cells were cultured in RPMI 1640 medium (Invitrogen) or Dulbecco's modified Eagle's medium (Invitrogen), respectively, with 10% fetal bovine serum (HyClone, Logan, UT), 1 mM L-glutamine, and 26 mM sodium bicarbonate for monolayer cell culture. The dishes containing cells were kept in a 37 °C humidified incubator with a mixture of 95% air and 5% CO_2. One day prior to the experiment, cells were plated in 60-mm dishes. For trypan blue exclusion assay (26), trypsinized cells were pelleted and resuspended in 0.2 ml of medium, 0.5 ml of 0.4% trypan blue solution, and 0.3 ml of phosphate-buffered saline solution (PBS). The samples were mixed thoroughly, incubated at room temperature for 15 min, and examined under a light microscope. At least 300 cells were counted for each survival determination.

Production of Recombinant TRAIL—A human TRAIL cDNA fragment (amino acids 114–281) obtained by RT-PCR was cloned into pET-23d (Novagen, Madison, WI) plasmid, and His-tagged TRAIL protein was purified using the QIAexpress protein purification system (Qiagen, Valencia, CA).

TUNEL Assay—For detection of apoptosis by the TUNEL method, cells were plated in slide chambers. After treatment, cells were fixed with 70% ethanol in PBS. Cells were washed once, permeabilized by incubating with 100 μl of 0.1% Triton X-100, 0.1% sodium citrate, and then washed twice in PBS. The TUNEL reaction was carried out at 37 °C for 1 h with 0.3 nmol of fluorescein isothiocyanate-12-dUTP, 3 nmol of dATP, 2 μl of $CoCl_2$, 25 units of terminal deoxynucleotidyltransferase, and TdT buffer (30 mM Tris, pH 7.2, 140 mM sodium cacodylate) in a total reaction volume of 50 μl. The reaction was stopped with 2 μl of 0.5 M EDTA. Cells were observed under a fluorescence microscope.

RNA Interference by siRNA of COX-2—To down-regulate the *COX-2*, *COX-2* siRNA (Santa Cruz Biotechnology) was used. *COX-2* siRNA was transfected into LNCaP cells and incubated for 36 h. The interference of COX-2 protein expression was confirmed by immunoblot using anti-COX-2 antibody (Cayman Chemical).

Transfection—In order to generate Bcl-2 overexpressing LNCaP cells and CX-1 cells, cells were transfected with pcDNA3-Bcl-2 or pcDNA3-neo using Lipofectamine Plus (Invitrogen). Transfected cells were selected for 3 weeks in growth medium containing 0.5 mg of G-418 (geneticin; Invitrogen) per ml. The clone expressing the highest level of Bcl-2 was used for this study. The expression level was determined by immunoblot analysis.

Protein Extracts and PAGE—Cells were lysed with 1× Laemmli lysis buffer (2.4 M glycerol, 0.14 M Tris, pH 6.8, 0.21 M SDS, 0.3 mM bromphenol blue) and boiled for 10 min. Protein content was measured with BCA Protein Assay Reagent (Pierce). The samples were diluted with 1× lysis buffer containing 1.28 M β-mercaptoethanol, and equal amounts of protein were loaded on 8–12% SDS-polyacrylamide gels. SDS-PAGE analysis was performed according to Laemmli (27) using a Hoefer gel apparatus.

Immunoblot Analysis—Proteins were separated by SDS-PAGE and electrophoretically transferred to nitrocellulose membrane. The nitrocellulose membrane was blocked with 5% nonfat dry milk in PBS/Tween 20 (0.1%, v/v) at 4 °C overnight. The membrane was incubated with primary antibody (diluted according to the manufacturer's instructions) for 2 h. Horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG was used as the secondary antibody. Immunoreactive protein was visualized by the chemiluminescence protocol (ECL, Amersham Biosciences).

In Vitro Kinase Assay—For immunocomplex kinase assay, cells were lysed with 500 μl of buffer A (20 mM Tris-HCl, pH 7.5, 1% Triton X-100, 0.5% deoxycholate, 5 mM EGTA, 150 mM NaCl, 10 mM NaF, 1 mM DTT, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and protein inhibitor mixture solution (Sigma)). Cell lysates were immunoprecipitated with anti-IKKα or anti-IKKβ antibody. Immune complexes were washed twice with buffer B (150 mM NaCl, 20 mM Tris-HCl, pH 7.5, 5 mM EGTA, 0.5 mM DTT, 1 mM Na_3VO_4, and 1 mM phenylmethylsulfonyl fluoride) at 4 °C and then incubated with 2 μg of purified GST-IκBα protein (Upstate Biotechnology) in the presence or absence of 1 mM ASA in a volume of 50 μl of a kinase buffer (100 μM ATP, 20 mM Tris-HCl, pH 7.5, 20 mM $MgCl_2$, 0.1 mM EDTA) for 30 min at 30 °C. Thereafter, the sample was subjected to SDS-PAGE, and the phosphorylation of GST-IκBα was analyzed by anti-phospho-IκBα antibody (Cell Signaling).

Measurement of Mitochondrial Membrane Depolarization—The mitochondria-specific dye TMRM (Molecular Probe, Eugene, OR) was used to measure the mitochondrial potential. CX-1 cells were grown in 6-well plates and were pretreated with 1 mM aspirin in the presence or absence of TRAIL (200 ng/ml). After treatment, the cells were collected, washed in PBS, and resuspended in 500 μl of FASC buffer. Cells were incubated for 20 min with 200 nM TMRM (Molecular Probe) at 4 °C in the dark, washed in cold PBS twice, and then resuspended in 500 μl of PBS buffer. The cells were visualized by flow cytometry. Positive samples were stained with carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (mitochondrial membrane-depolarized inducer), and the surface markers were analyzed by an EPICS XL-MCL flow cytometer with a single argon laser at 488 nm (Beckman Coulter, Inc., Hialeah, FL).

Isolation of Nuclear Proteins—Nuclear extracts were prepared by the modified procedure of Dignam *et al.* (28). Following treatment with ASA for 20 h, LNCaP cells were washed three times with PBS and incubated on ice for 15 min in hypotonic buffer A (10 mmol/liter HEPES, pH 7.9, 10 mmol/liter KCl, 0.1 mmol/liter EDTA, 0.1 mmol/liter EGTA, 1 mmol/liter DTT, 0.5 mmol/liter phenylmethylsulfonyl fluoride, and 0.6% Nonidet P-40). Cells were vortexed gently for lysis, and the nuclei were separated from the cytosol by centrifugation at 12,000 × *g* for 1 min. Nuclei were resuspended in buffer C (20 mmol/liter HEPES, pH 7.9, 25% glycerol, 0.4 mol/liter NaCl, 1 mmol/liter EDTA, 1 mmol/liter EGTA, 1 mmol/liter DTT, and 0.5 mmol/liter phenylmethylsulfonyl fluoride) and shaken for 30 min at 4 °C. Nuclear extracts were obtained by centrifugation at 12,000 × *g*, and protein concentration was measured by Bradford assay (Bio-Rad). NF-κB in nuclear extracts was detected by Western blotting as described above.

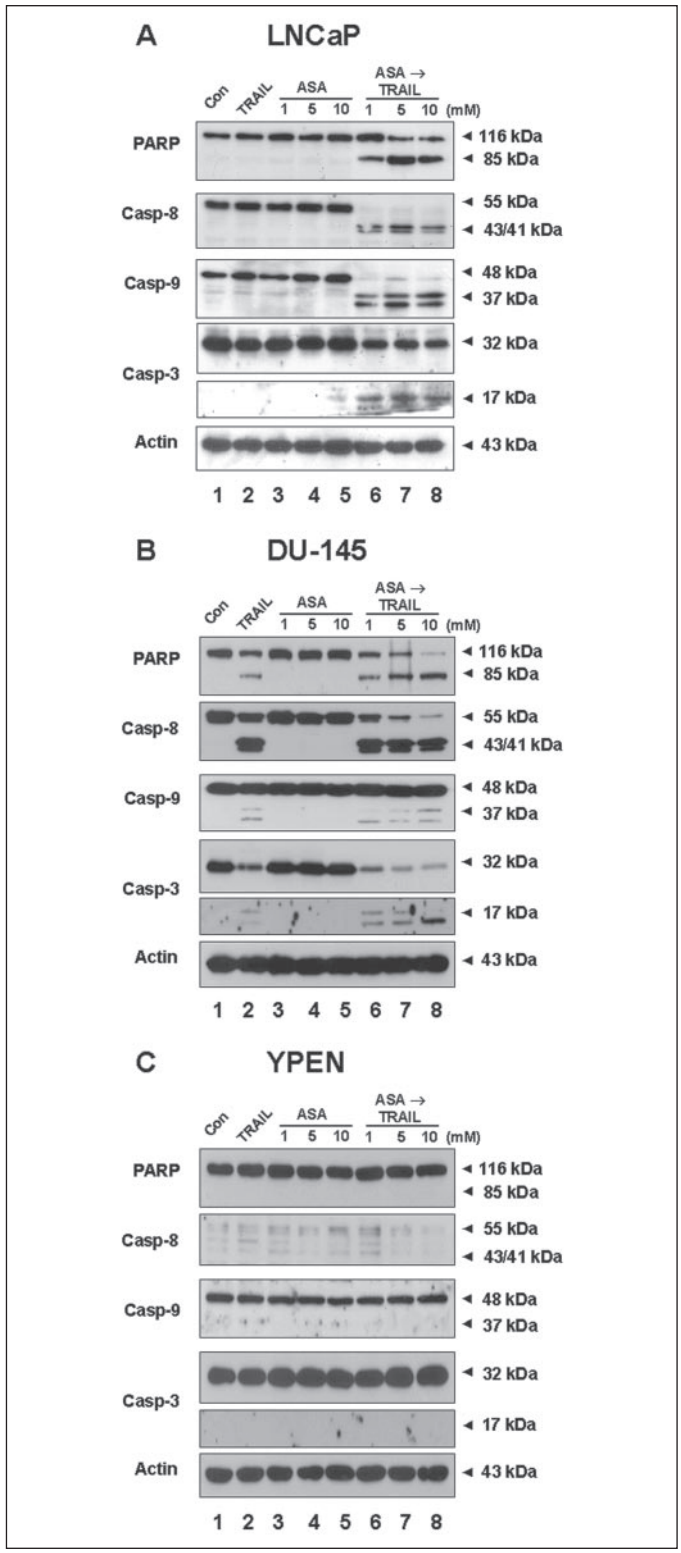

FIGURE 2. **Effect of pretreatment of acetylsalicylic acid on TRAIL-induced proteolytic cleavage of PARP and activation of caspases in LNCaP (*A*), DU-145 (*B*), or YPEN (*C*) cells.** Cells were pretreated with various concentrations of ASA (1–10 mM) for 20 h, treated with/without TRAIL (200 ng/ml in LNCaP and YPEN, 50 ng/ml in DU-145) for 4 h, and then harvested. Cell lysates were subjected to immunoblotting for PARP, caspase-8, caspase-9, or caspase-3. Antibody against caspase-8 detects inactive form (55 kDa) and cleaved intermediates (41 and 43 kDa). Anti-caspase-9 antibody detects both inactive form (48 kDa) and cleaved intermediate (37 kDa). Anti-caspase-3 antibody detects inactive form (32 kDa) and cleaved active form (17 kDa). Immunoblots of PARP show the 116-kDa PARP and the 85-kDa apoptosis-related cleavage fragment. Actin was used to confirm the amount of proteins loaded in each lane. *Con*, control; *Casp*, caspase.

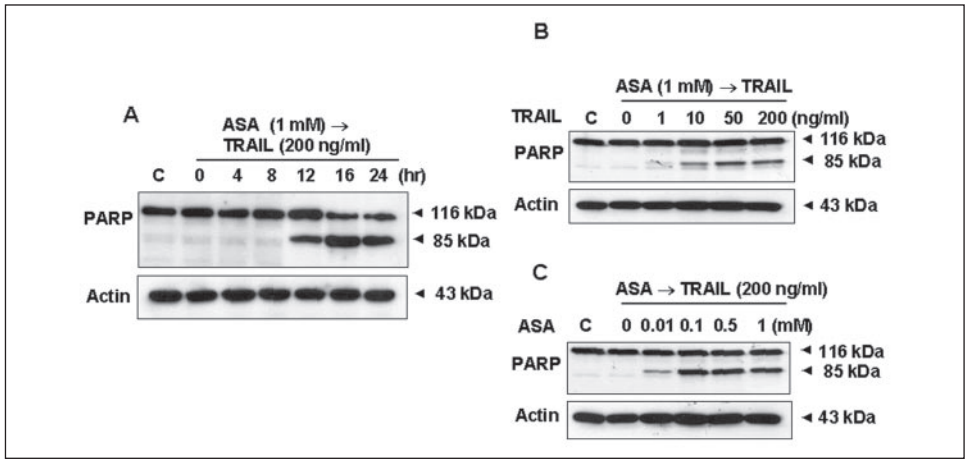

FIGURE 3. **Effect of pretreatment of acetylsalicylic acid on TRAIL-induced PARP cleavage in LNCaP cells.** *A,* cells were pretreated with 1 mM ASA for various times (0–24 h) and treated with 200 ng/ml TRAIL for 4 h. *B,* cells were pretreated with 1 mM ASA for 20 h and treated with various concentrations of TRAIL (1–200 ng/ml) for 4 h. *C,* cells were treated with various concentrations of ASA (0.01–1 mM) for 20 h and treated with 200 ng/ml TRAIL for 4 h. Equal amounts of protein (20 μg) were separated by SDS-PAGE and immunoblotted with anti-PARP antibody. Actin is shown as an internal standard. *Lane C*, control.

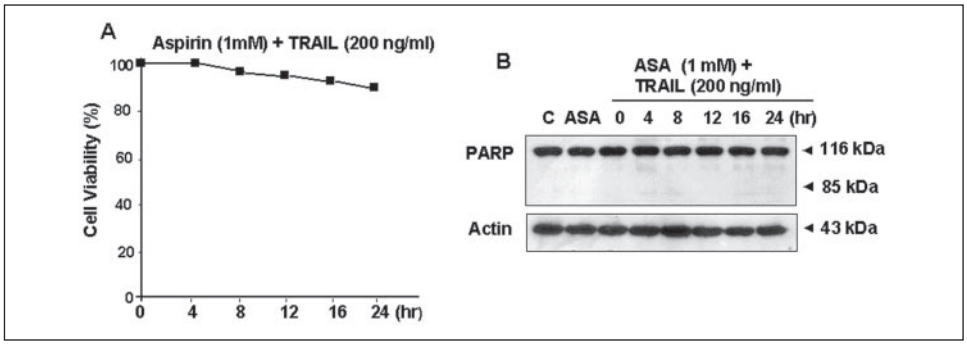

FIGURE 4. **TRAIL in combination with acetylsalicylic acid without pretreatment of acetylsalicylic acid has no effect on TRAIL-induced apoptosis in LNCaP cells.** *A,* cells were treated with 200 ng/ml TRAIL in combination with 1 mM ASA for various times (0–24 h). Cell survival was determined by the trypan blue exclusion assay. *Error bars* represent the means ± S.E. from three separate experiments. *B,* equal amounts of protein (20 μg) were separated by SDS-PAGE and immunoblotted with anti-PARP antibody. Actin is shown as an internal standard. *Lane C*, control.

Electrophoretic Mobility Shift Assay—LNCaP cells were treated with various concentrations of ASA (0.01–1 mM) or 200 ng/ml TRAIL for 20 h, and nuclear extract was prepared as described above. The nuclear extract (10 μg of protein) was incubated with binding buffer (20 mM HEPES, pH 7.6, 1 mM EDTA, 1 mM ammonium sulfate, 1 mM DTT, 30 mM KCl, 0.2% Tween 20) and 1 μg of poly(dI-dC) for 10 min on ice. Biotin-labeled probe NF-κB-specific oligonucleotide (5′-AGTTGAGGGGACTTTCCCAGGC-3′) was used. The reaction was incubated at room temperature for 30 min. The negative control consisted of free probe only. A competition control was set up by adding non-biotin-labeled cold probe to the reaction. The samples separated on a 6% native polyacrylamide gel in 0.5% TBE for 50 min at 120 V. The samples were then transferred in 0.5% TBE onto a nylon membrane at 300 mA for 40 min. After transfer, the sample was fixed on the membrane by UV cross-linking. The membrane was first blocked with 1% blocking reagent (Roche Applied Science) at room temperature for 30 min. The biotin-labeled probe was then detected with streptavidin-horseradish peroxidase diluted 1: 20,000 (Pierce). After washing three times and equilibrating in buffer, the membrane was overlaid with lumino/enhancer and substrate for 5 min. The image was acquired using a Kodak X-Omat 2000A (Eastman Kodak, Rochester, NY).

Flow Cytometry—Cells were treated with ASA for the indicated time points with or without TRAIL. After washing, cells were blocked for 30 min with 1% bovine serum albumin in PBS. Cells were then incubated with 1 μg of primary antibodies to DR4 or DR5 (Alexis) in 1% bovine serum albumin for 30 min followed by washing with PBS. Finally, cells were incubated with Alexa 488-conjugated goat anti-mouse IgG (Molecular Probe) for 30 min. After washing, the cells were analyzed on a FACScan flow cytometer. Matched isotype using control IgG antibodies was included.

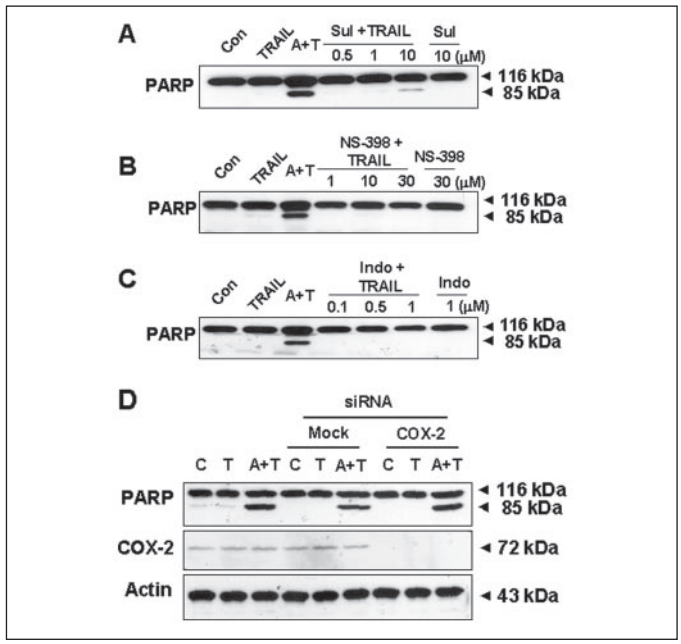

FIGURE 5. **Role of COX in acetylsalicylic acid-induced TRAIL cytotoxicity.** *A–C,* LNCaP cells were pretreated with 1 mM ASA (*A* + *T*), or various concentrations of sulindac sulfide (*Sul* + *TRAIL*), NS-398 (*NS-398* + *TRAIL*), or indomethacin (*Indo* + *TRAIL*) for 20 h and treated with 200 ng/ml TRAIL for 4 h. Equal amounts of protein (20 μg) were separated by SDS-PAGE and immunoblotted with anti-PARP antibody. *D,* LNCaP cells were transfected with *COX-2* siRNA or mock siRNA and incubated for 36 h. Cells were pretreated with 1 mM ASA for 20 h and treated with 200 ng/ml TRAIL for 4 h. Equal amounts of protein (20 μg) were separated by SDS-PAGE and immunoblotted with anti-PARP, anti-COX-2, or anti-actin antibody. *C,* untreated control cells; *T,* TRAIL-treated cells; *A*+*T,* ASA- and TRAIL-treated cells.

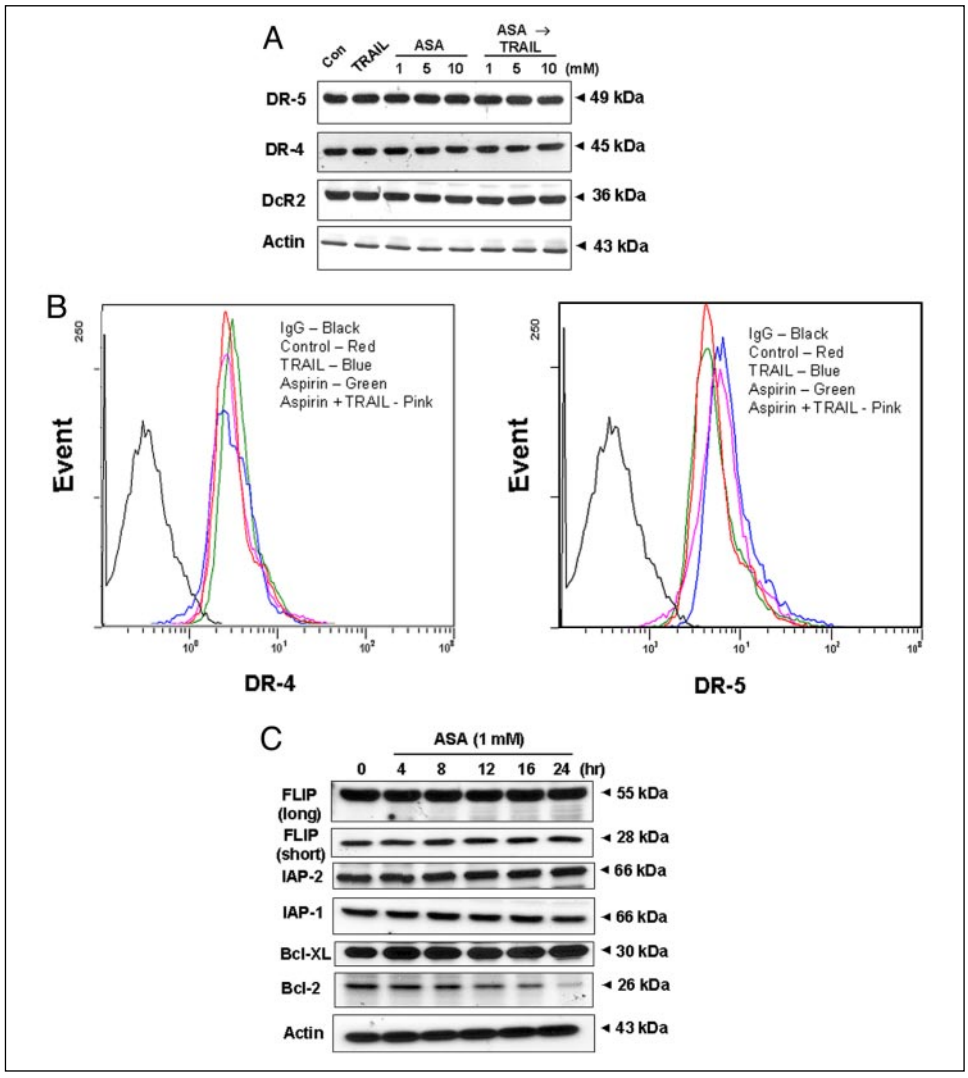

FIGURE 6. **Effect of pretreatment of acetylsalicylic acid in combination with TRAIL on intracellular levels of TRAIL receptors (*A*), expression of functional TRAIL receptors on membrane (*B*), or anti-apoptotic proteins (*C*) in LNCaP cells.** *A* and *B,* cells were pretreated with various concentrations of ASA (1–10 mM) for 20 h and treated with/without 200 ng/ml TRAIL for 4 h. *A,* equal amounts of protein (20 μg) were separated by SDS-PAGE and immunoblotted with anti-DR-5, anti-DR-4, anti-DcR2, or anti-actin antibody. Actin was shown as an internal standard. *Con,* control. *B,* cells were stained with Alexa 488-conjugated antibodies and analyzed with a FACScan flow cytometer. Staining with isotype-matched control IgG represents negative control. *C,* cells were treated for various times (4–24 h) with 1 mM ASA and harvested. Equal amounts of protein (20 μg) were separated by SDS-PAGE and immunoblotted with anti-FLIP, anti-IAP-1, anti-IAP-2, anti-Bcl-XL, anti-Bcl-2, or anti-actin antibody. Actin is shown as an internal standard.

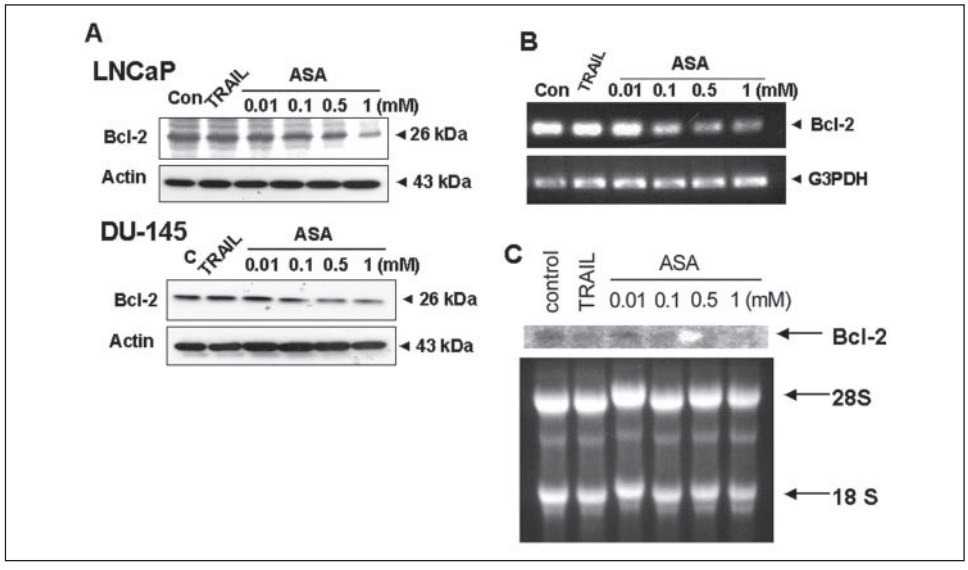

FIGURE 7. **Acetylsalicylic acid-mediated down-regulation of Bcl-2 expression in LNCaP or DU-145 cells.** *A,* cells were treated with various concentrations of ASA (0.01–1 mM) or 200 ng/ml TRAIL for 20 h for LNCaP cells or 10 ng/ml TRAIL for 20 h for DU-145 cells. Equal amounts of protein (20 μg) were separated by SDS-PAGE and immunoblotted with anti-Bcl-2 or anti-actin antibody. Actin was shown as an internal standard. *B,* RT-PCR analysis was performed for detecting *BCL-2* or glyceraldehyde-3-phosphate dehydrogenase (*G3PDH*) expression in LNCaP cells. Glyceraldehyde-3-phosphate dehydrogenase is shown as an internal standard. *C,* Northern blot analysis was performed for detecting Bcl-2. Total electrophoretically fractionated RNA was probed with the *BCL-2* cDNA fragment (*upper panel*). The ethidium bromide-stained ribosomal RNAs are shown as a loading control (*lower panel*). *Con,* control.

RT-PCR Analysis of Bcl-2 mRNA Levels—Total cellular RNA was extracted using the Trizol method (Invitrogen) according to the manufacturer's instructions. For each RT-PCR, 1 μg of total RNA was used with Novagen One-step RT-PCR kit (EMD Bioscience). The following sense and antisense primers were used at 0.5 μM for each: Bcl-2 primer, sense, 5′-CGACGACTTCTCCCGCCGCTACCGC-3, and antisense,

5′-CCGCATGCTGGGGCCGTACAGTTCC-3′; glyceraldehyde-3-phosphate dehydrogenase primer, sense, 5′-TCCACCACCCTGTTGCTGTA-3′, and antisense, 5′-ACCACAGTC-CATGCCATCAC-3′. The reaction conditions were 40 cycles at reverse transcription at 60 °C for 30 min, initial PCR activation at 94 °C for 2 min, denature at 94 °C for 1 min, anneal at 60 °C for 90 s, and final extension at 60 °C for 7 min. After amplification, the products were resolved by electrophoresis on 1% agarose gel, stained with ethidium bromide, and photographed under ultraviolet light.

Northern Blot Analysis—LNCaP cells were treated with either 200 ng/ml TRAIL or various concentrations of aspirin (0.01, 0.1, 0.5, and 1 mM) for 20 h. For Northern blot hybridization, total RNAs (10 μg) isolated from the above-treated cells were fractionated by electrophoresis in formaldehyde-1.2% agarose gels, blotted onto Nytran Plus (Schleicher & Schuell), and hybridized with the ^{32}P-labeled *BCL-2* cDNA probe. The 448-bp *BCL-2* cDNA probe was amplified by using PCR with primers 5′-CACCTGACCCTCCGCCAG-3′ (forward) and 5′-TGTTGACTTCACTTGTGGCCCAG-3′ (reverse).

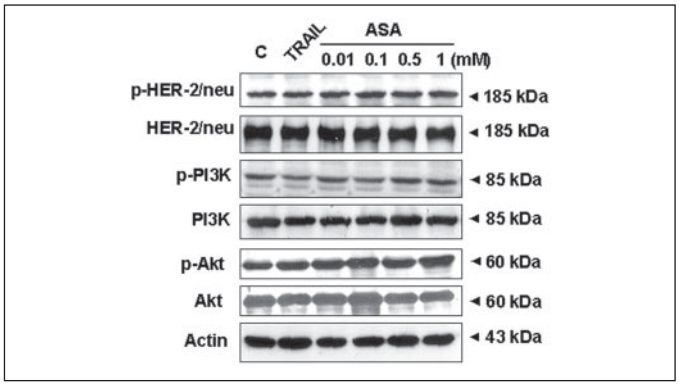

FIGURE 8. **Effect of acetylsalicylic acid on the HER-2/neu-PI3K-Akt signal transduction pathway in LNCaP cells.** Cells were treated with various concentrations of ASA (0.01–1 mM) or 200 ng/ml TRAIL for 20 h. Equal amounts of protein (20 μg) were separated by SDS-PAGE and immunoblotted with anti-HER-2/neu, anti-phospho-HER-2/neu, anti-PI3K, anti-phospho-PI3K, anti-Akt, anti-phospho-Akt, or anti-actin antibody. *C*, control.

RESULTS

ASA Promotes TRAIL-induced Cytotoxicity—To investigate the effect of ASA on TRAIL-induced cytotoxicity, human prostatic adenocarcinoma LNCaP cells were pretreated with ASA and treated with TRAIL in the presence of ASA. Fig. 1, *A* and *B*, shows that little or no cytotoxicity was observed with 1 mM ASA alone or 200 ng/ml TRAIL alone. However, pretreatment of ASA promoted TRAIL-induced cytotoxicity that was dependent upon concentrations of ASA (Fig. 1*A*) and TRAIL (Fig. 1*B*). Similar results were observed with TUNEL staining (Fig. 1*C*). Data from TUNEL assays show that apoptotic cell death occurred when LNCaP cells were pretreated with ASA followed by TRAIL.

Effect of ASA on TRAIL-induced Apoptosis—Additional studies were designed to examine whether pretreatment with ASA followed by treatment with TRAIL causes PARP cleavage, the hallmark feature of apoptosis, in prostate cancer LNCaP and DU-145 cells and normal prostate YPEN cells. Fig. 2 shows that PARP (116 kDa) was cleaved yielding a characteristic 85-kDa fragment in the presence of TRAIL (50–200 ng/ml) and ASA (1–10 mM) in prostate cancer cells, but not in normal prostate cells. The cleavage of PARP was not observed by treatment with ASA alone. These results were similar to the observations of cytotoxicity (Fig. 1, *A* and *B*). Western blot analysis shows that procaspase-8 (55 kDa) was cleaved to the intermediates (41 and 43 kDa) by pretreatment with ASA and treatment with TRAIL in LNCaP and DU-145 cells. The combined treatment of TRAIL and ASA also resulted in an increase in caspase-9 activation as well as caspase-3 activation in LNCaP and DU-145 cells (Fig. 2, *A* and *B*). The precursor form of caspase-9 and -3 was cleaved to the active form of 37 and 17 kDa, respectively. ASA alone did not activate caspases. We extended our studies to investigate a time course and dose response on PARP cleavage. Fig. 3*A* shows that at least 12 h of pretreatment with ASA was required for PARP cleavage in the presence of TRAIL. Fig. 3, *B* and *C*, shows that a minimal amount of 10 ng/ml TRAIL or 0.01 mM ASA was required for PARP cleavage in the presence of 1 mM ASA or 200 ng/ml TRAIL, respectively, in LNCaP cells. We further investigated whether treatment with ASA is a prerequisite. Fig. 4 shows that combined treatment with TRAIL and ASA without pretreatment with ASA caused little or no cytotoxicity and PARP cleavage. Taken together, these results suggest that pretreatment

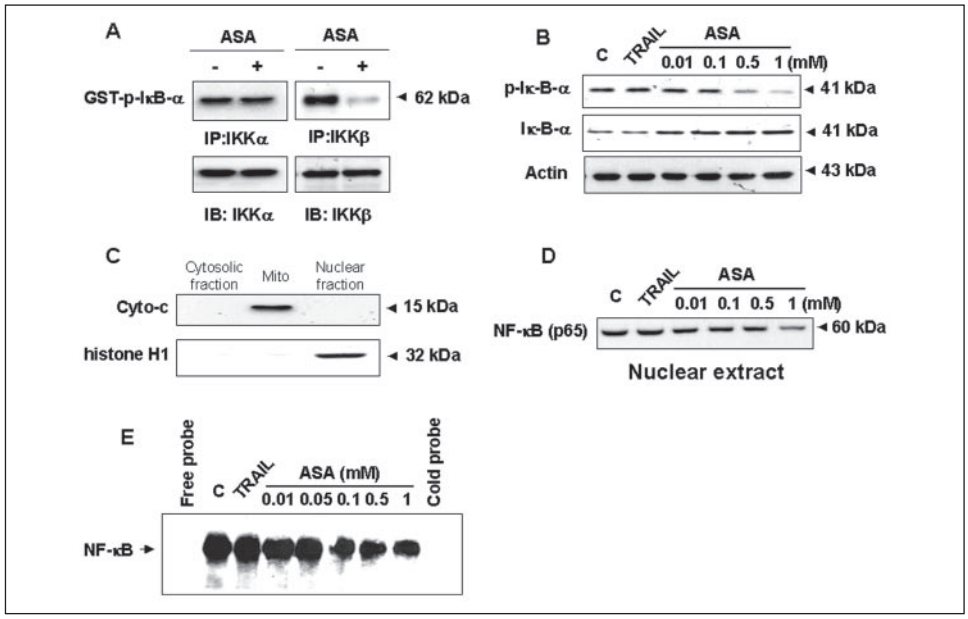

FIGURE 9. **Effect of acetylsalicylic acid on IKK activity (*A*), IκB-α phosphorylation (*B*), or NF-κB (*D* and *E*) translocation in LNCaP cells, and purity of the nuclear extracts (*C*).** *A*, cells were lysed, and IKK proteins were purified by immunoprecipitation (*IP*). The purified IKK proteins were incubated with or without 1 mM ASA for 30 min at 4 °C, and *in vitro* kinase assay was performed at 30 °C for 30 min with GST-IκB-α as substrate. *B*, cells were treated with various concentrations of ASA (0.01–1 mM) or 200 ng/ml TRAIL for 20 h and lysed. Equal amounts of protein (20 μg) were separated by SDS-PAGE and immunoblotted (*IB*) with anti-phospho-IκB-α, anti-IκB-α, or anti-actin antibody. *Lane C*, control. *C–E*, cells were treated with various concentrations of ASA (0.01–1 mM) or 200 ng/ml TRAIL for 20 h, and nuclear proteins were extracted (*C*). *D*, equal amounts of nuclear protein (20 μg) were separated by SDS-PAGE and immunoblotted with anti-NF-κB antibody. *E*, the nuclear extracts were incubated with biotin-labeled oligonucleotide at room temperature for 30 min. Gel mobility shift assays were performed as described under the "Experimental Procedures."

with ASA for 12 h is essential for inducing apoptotic death in the presence of TRAIL.

Role of COX in TRAIL-induced Apoptosis—It is well known that ASA inhibits only COX-1 at low concentrations (IC_{50} = 44 μM) but both COX-1 and COX-2 at higher concentrations (IC_{50} = 1100 μM) (29). To examine whether the promotive effect of ASA on TRAIL-induced apoptosis is mediated through inhibiting COX, LNCaP cells were pretreated with various NSAIDs and then treated with TRAIL. Unlike ASA, Fig. 5, *A–C,* shows that no significant cleavage of PARP was observed by treatment with various concentrations of sulindac sulfide (IC_{50} = 1.02 μM for COX-1 and IC_{50} = 10.43 μM for COX-2), NS-398 (a selective COX-2 inhibitor; IC_{50} = 4.81 μM for COX-1 and IC_{50} = 0.47 μM for COX-2), or indomethacin (a nonselective COX inhibitor; IC_{50} = 0.16 μM for COX-1 and IC_{50} = 0.46 μM for COX-2). To confirm our observations, cells were transfected with *COX-2* siRNA or mock siRNA. Fig. 5*D* shows that the expression of *COX-2* was effectively inhibited by si*COX-2*. However, knock-down of *COX-2* expression did not promote TRAIL-induced apoptosis. Nonetheless, pretreatment with ASA promoted TRAIL-induced apoptosis regardless of the presence or the absence of COX-2. These results suggest that COX is not involved in ASA-promoted TRAIL cytotoxicity.

Effect of ASA on the Level of TRAIL Receptor Family and Anti-apoptotic Proteins—It is well known that TRAIL can interact with death receptors (DR4 and DR5), which trigger apoptotic signals (4). Such signals may be blocked by expression of the antagonistic decoy receptors (DcR1 and DcR2). Previous studies demonstrate that increased DR5 levels are induced by chemotherapeutic agents (30). Thus, we examined whether pretreatment with ASA affects the level of TRAIL receptors and anti-apoptotic proteins, and consequently promotes apoptosis by treatment with TRAIL. LNCaP cells were pretreated with ASA (1–10 mM) and treated with 200 ng/ml TRAIL in the presence of ASA. Data from Western blot analysis reveal that ASA treatment did not significantly alter the total cellular levels of the TRAIL receptors (DR4, DR5, and DcR2) and anti-apoptotic proteins ($FLIP_L$, $FLIP_S$, IAP-1, IAP-2, and Bcl-$X_{L)}$ (Fig. 6, *A* and *C*). Data from flow cytometric analysis show that TRAIL induced surface expression of DR5 but not DR4 (Fig. 6*B*). However, ASA treatment did not enhance the DR5 expression. Most interestingly, ASA treatment resulted in a decrease in the level of Bcl-2 (Fig. 6*C*). The reduction of Bcl-2 during treatment with 1 mM ASA was dependent upon exposure time (Fig. 6*B*). To confirm the effect of ASA on *BCL-2* gene expression, LNCaP or DU-145 prostatic cancer cells were treated with various concentrations of ASA, and expression of *BCL-2* was examined. Fig. 7*A* shows that ASA reduced the level of Bcl-2 in both cell lines. Data from RT-PCR and Northern blot assay in Fig. 7, *B* and *C,* show that the level of *BCL-2* mRNA was significantly decreased during treatment with ASA. The reduction of BCL-2 mRNA was dependent upon ASA concentration. These results suggest that the reduction of Bcl-2 levels during treatment with ASA was because of suppression of *BCL-2* gene transcription.

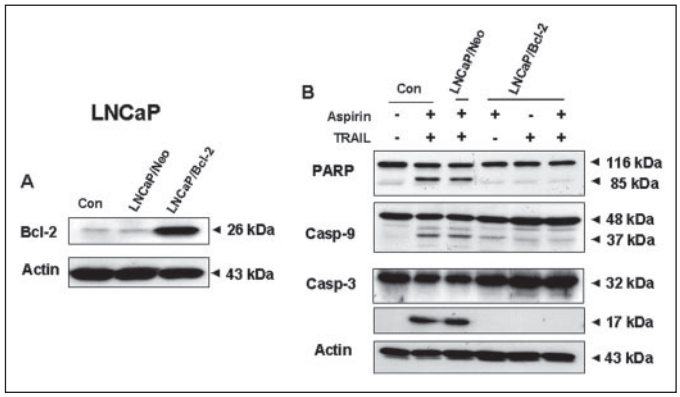

FIGURE 10. **Overexpression of *BCL-2* effectively inhibits potentiation of TRAIL-induced apoptosis by acetylsalicylic acid in LNCaP cells.** *A,* immunoblot of *BCL-2* expression in control vector-transfected (LNCaP/neo) and *BCL-2* transfected (LNCaP/Bcl-2) single cell clones of LNCaP cells. *Con,* untransfected parental control cells. *B,* parental control, LNCaP/neo, or LNCaP/Bcl-2 cells were treated with 200 ng/ml TRAIL for 4 h with/without pretreatment of 1 mM ASA for 20 h and then harvested. Cell lysates were subjected to immunoblotting for caspase (*Casp*)-9, caspase-3, PARP, or actin. Actin is shown as an internal standard.

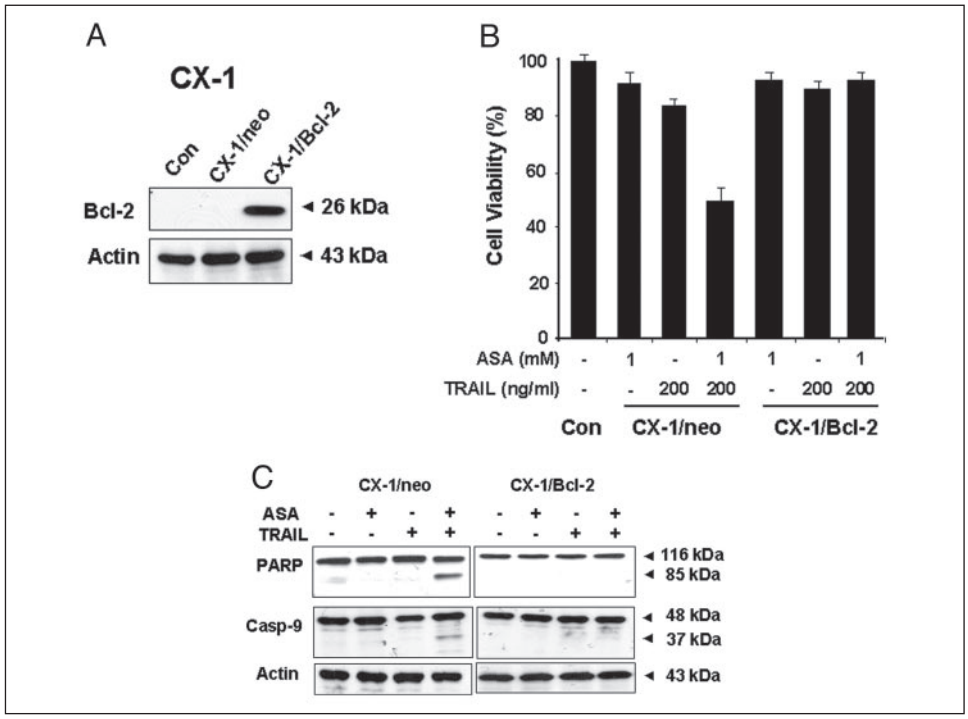

FIGURE 11. **Overexpression of *BCL-2* effectively inhibits augmentation of TRAIL-induced apoptotic death by acetylsalicylic acid in colorectal carcinoma CX-1 cells.** *A,* immunoblot of *BCL-2* expression in control vector-transfected (CX-1/neo) and *BCL-2*-transfected (CX-1/Bcl-2) single cell clones of CX-1 cells. *Con,* untransfected parental control cells. *B,* cells were pretreated with 1 mM ASA for 20 h and treated with 200 ng/ml TRAIL for 4 h. Cell survival was determined by the trypan blue exclusion assay. *Error bars* represent the mean ± S.E. from three separate experiments. *C,* parental control, CX-1/Neo, or CX-1/Bcl-2 cells were treated with 200 ng/ml TRAIL for 4 h with/without pretreatment of 1 mM ASA for 20 h and then harvested. Cell lysates were subjected to immunoblotting for caspase (*Casp*)-9, PARP, or actin. Actin is shown as an internal standard.

Effect of ASA on the HER-2/neu-PI3K-Akt-NF-κB Signal Transduction Pathway—It is well known that *BCL-2* expression is regulated by NF-κB, a dimeric transcription factor (31). We postulated that ASA inhibits NF-κB activity, which subsequently decreases transcription of *BCL-2*. To examine this possibility, the effect of ASA on upstream signal transduction of NF-κB was investigated. Fig. 8 shows that ASA treatment did not change the level of HER-2/neu, PI3K, and Akt or alter the phosphorylation of these proteins. In contrast, ASA treatment inhibited IKKβ activity, dephosphorylated IκB-α, increased the level of IκB-α, and prevented NF-κB nuclear translocation (Fig. 9). These results suggest that ASA down-regulates *BCL-2* gene expression by inhibiting the IKKβ-IκB-α-NF-κB signal transduction pathway.

Role of Bcl-2 in ASA-enhanced TRAIL Cytotoxicity—To determine whether ASA-mediated down-regulation of *BCL-2* plays an important role in the augmentation of TRAIL-induced apoptotic death, LNCaP cells or human colorectal carcinoma CX-1 cells were stably transfected with either an empty control vector (pcDNA 3-neo) or vector containing *BCL-2* (pcDNA3-Bcl-2). Figs. 10 and 11 show that pretreatment with ASA followed by treatment with TRAIL caused PARP cleavage, activation of caspases, as well as cytotoxicity in control vector transfected cells. However, overexpression of *BCL-2* protected LNCaP and CX-1 cells from ASA-enhanced TRAIL cytotoxicity. These results suggest that ASA-promoted TRAIL cytotoxicity is mediated by down-regulating *BCL-2*.

Overexpression of BCL-2 Prevents Alteration of Mitochondrial Membrane Potential by Treatment with ASA and TRAIL—Bcl-2 is an anti-apoptotic protein that inhibits the release of cytochrome *c* from mitochondria into the cytoplasm, thereby down-regulation of *BCL-2* may promote intrinsic mitochondria-mediated apoptosis (32, 33). To investigate whether ASA disrupts mitochondrial membrane potential and overexpression of *BCL-2* protects cells from this disruption, CX-1/Bcl-2 or CX-1/neo cells were pretreated with ASA and treated with TRAIL. We used the mitochondria-specific dye TMRM to measure the mitochondrial membrane potential. Fig. 12 shows that overexpression of *BCL-2* inhibited the loss of mitochondrial membrane potential during treatment with ASA alone or ASA in combination with TRAIL.

A Model for the Effect of ASA on the TRAIL-induced Apoptotic Pathway—Fig. 13 shows a schematic diagram of a model that is based on the literature and our data. ASA blocks the Akt-NF-κB survival signal pathway by inhibiting IKKβ. The inhibition of this pathway results in suppression of the expression of *BCL-2*, an anti-apoptotic molecule.

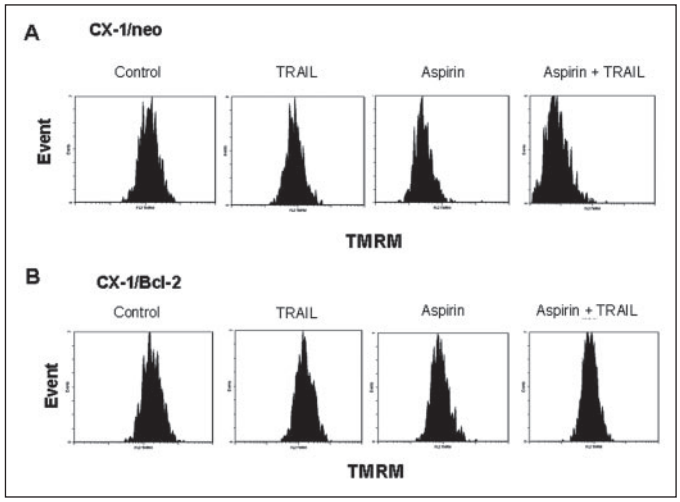

FIGURE 12. **Effect of *BCL-2* overexpression on acetylsalicylic acid and TRAIL-induced mitochondrial membrane perturbations.** CX-1/neo (*A*) or CX-1/Bcl-2 (*B*) cells were pretreated with 1 mM ASA for 20 h and treated with/without 200 ng/ml TRAIL for 4 h. Mitochondrial membrane potential was assessed by flow cytometry using the fluorescent dye TMRM.

DISCUSSION

Aspirin (acetylsalicylic acid) is a nonsteroidal anti-inflammatory drug (NSAID) widely used for its anti-pyretic and analgesic properties. ASA is also known to induce gastrointestinal side effects, mainly in the form of gastric and duodenal ulcerations or erosions. However, epidemiological findings have revealed that ASA reduces the risk of colorectal cancer and adenoma (34, 35). In this study, we demonstrate that pretreatment with ASA promotes TRAIL-induced apoptotic death. The enhancement of apoptosis by treatment with ASA is probably because of down-regulation of *BCL-2*, activation of caspases, induction of conformational change, translocation of Bax, and cytochrome *c* release (Figs. 2, 6, and 7) (36–40). Our observations were similar to previous reports (41). Previous studies have shown that aspirin inhibits the transcription factor

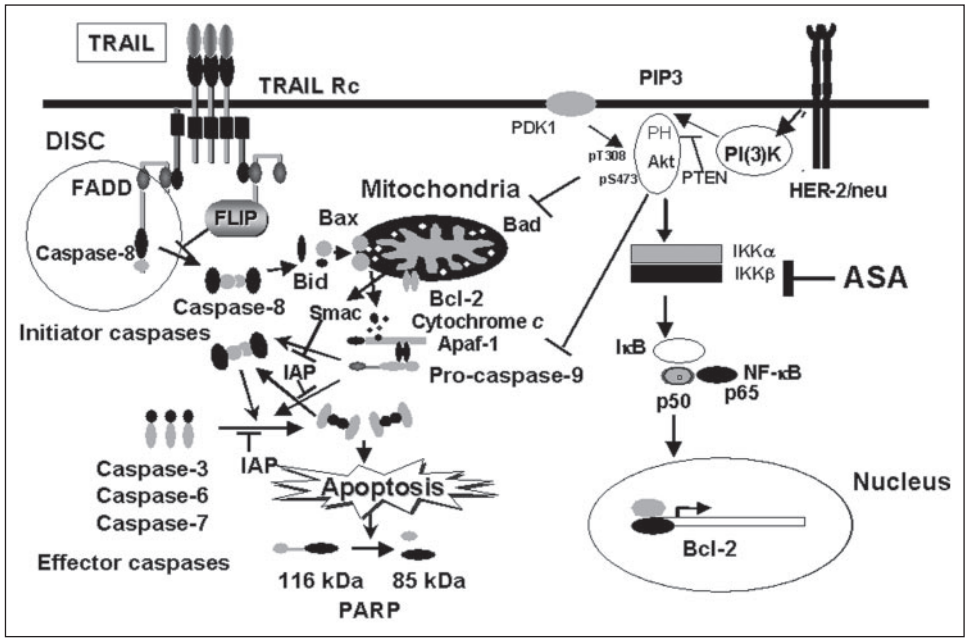

FIGURE 13. **A schematic model for the effect of acetylsalicylic acid on the TRAIL-induced apoptotic death signal pathway.** *PIP_3*, phosphatidylinositol 3,4,5-triphosphate; *PH*, pleckstrin homology.

NF-κB (42, 43), which is critical for the expression of several anti-apoptotic genes including *C-IAP1*, *C-IAP2*, *BCL-X_L*, *FLIP*, and *BCL-2* (31, 44–46). The inhibition of NF-κB activity is mediated through preventing the phosphorylation and degradation of the inhibitory subunit IκB (Fig. 9) (47). Although the expression of these *Bcl-2* family and IAP family proteins is known to be regulated by NF-κB, our data show that ASA inhibits preferentially *BCL-2* gene expression (Fig. 6). Thus, a fundamental question that remains unanswered is how ASA inhibits selectively the expression of the *BCL-2* gene among *BCL-2* family and *IAP* family genes. It is well known that the NF-κB family of proteins, including NF-κB1, NF-κB2, RelA, RelB, and c-Rel, can form homo- and heterodimers *in vitro*, except for RelB. In mammals, the most widely distributed NF-κB is a heterodimer composed of p50 and p65 (also called RelA) subunits (48). NF-κB activity is regulated by the IκB family of proteins that interacts with and sequesters the transcription factor in the cytoplasm. IκB proteins become phosphorylated by the multisubunit IKK complex, which subsequently targets IκB for ubiquitination and degradation by the 26 S proteasome (49). At this time only speculations can be made concerning the role of NF-κB in the down-regulation of *BCL-2* gene expression during treatment with ASA. One possibility is that differential activation of NF-κB may be responsible for selective inhibition of *BCL-2* gene expression. As mentioned above, the inhibition of five members of the NF-κB family may differ during treatment with ASA, and this differential inhibition of the various members of NF-κB family causes a selective inhibition of *BCL-2* gene expression (50). We believe that many critical questions still remain to be answered in order to understand the mechanisms of the regulation of *BCL-2* gene expression by ASA. However, this model will also provide a framework for future studies.

Previous studies have shown that ASA inhibits tumor necrosis factor-α- and interleukin-1-induced NF-κB activation and sensitizes HeLa cells to apoptosis (51). In this study, we observed that ASA augments TRAIL cytotoxicity in TRAIL-resistant LNCaP cells that contain high levels of HER-2/neu. It is well known that HER-2/neu has an intrinsic tyrosine kinase activity that activates PI3K in the absence of ligand (52). PI3K consists of a regulatory subunit (p85) that binds to an activated growth factor/cytokine receptor and undergoes phosphorylation, which results in the activation of its catalytic subunit (p110) (53). PI3K phosphorylates phosphoinositides at the 3′-position of the inositol ring, and its major lipid product is phosphatidylinositol 3,4,5-triphosphate (54). Phosphatidylinositol 3,4,5-triphosphate facilitates the recruitment of Akt to the plasma membrane through binding with the pleckstrin homology domain of Akt (54). Akt is activated by phosphoinositide-dependent kinase-1 (PDK1) through phosphorylation at threonine 308 and serine 473 (55). A number of pro-apoptotic proteins have been identified as direct Akt substrates, including BAD, caspase-9, and Forkhead transcription factors (56–61). The pro-apoptotic function of these molecules is suppressed upon phosphorylation by Akt. Recent studies also show that Akt induces the degradation of IκB by promoting IKKα activity and subsequently stimulating the nuclear translocation of NF-κB (62). In this study, we have revealed that ASA does not affect the HER-2/neu-PI3K-Akt signal transduction pathway (Fig. 8). However, ASA can interrupt the Akt-NF-κB signal transduction pathway by inhibiting IKKβ activity (Fig. 9) (47). A previous study shows that 1 mM aspirin treatment inhibits 75% of endogenous IKK kinase activity, even though more than 90% of IKKβ activity is inhibited without altering IKKα activity in the presence of aspirin (47). These results suggest that a small percentage of total IKKα forms IKKα homodimers that still contain kinase activity in the presence of aspirin. In this study, we believe that blockade of HER-2/neu-mediated survival signals by inhibiting IKKβ activity can sensitize TRAIL-resistant tumor cells. We also believe that this study will provide information to improve the efficacy of TRAIL-based clinical therapy.

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